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TITLE: Role of TGR-B1-Mediated Down Regulation of NF-kB/Rel
Activity During Growth Arrest of Breast Cancer Cells

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FOREWORD

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Shangji Guo 04/30/01
PI - Signature Date

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Appendix: One paper

*Please note that the PI of this grant has been changed from Dong Wook Kim to Shangqin Guo.

ABSTRACT

The NF- κ B/Rel family of dimeric transcription factors has been shown to promote cell survival, and increasing evidence suggests involvement in carcinogenesis. Recently, NF- κ B/Rel was found to be constitutively active in the nuclei of human breast cancer cell lines, as well as in 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors from Sprague-Dawley rats (S-D). Malignantly transformed human mammary epithelial cells (HMEC), derived by carcinogen treatment of non-tumorigenic parental MCF-10F cells displayed increased constitutive NF- κ B activation. In premalignant HMECs immortalized by carcinogen treatment *in vitro*, NF- κ B activity was dysregulated in quiescence. Furthermore, founder lines of transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established. Studies, which are still in progress, have indicated the appearance of breast tumors appear by an average of 19 months, thus confirming the role of NF- κ B/Rel in the pathogenesis of the mammary gland. Furthermore, our laboratory demonstrated activation of NF- κ B is induced upon over-expression of Her-2/neu. Thus, studies were initiated with green tea polyphenol, epigallocatechin-3 gallate (EGCG), which has been shown to inhibit the induction of NF- κ B and growth of breast cancer cell lines *in vitro*. EGCG reduced NF- κ B levels in the NF639 cell line derived from an MMTV-Her-2/neu mouse tumor. NF639 clonal isolates resistant to EGCG appear to display elevated levels of NF- κ B. Overall our studies provide evidence for involvement of the NF- κ B/Rel in malignant progression of mammary epithelial cells.

I. Introduction

NF- κ B/Rel is a family of transcription factors, which are expressed in all cells; however, in most non-B cells, they are sequestered in the cytoplasm in inactive complexes with specific inhibitory proteins, termed I κ Bs. We have recently shown that NF- κ B/Rel factors are aberrantly activated in breast cancer, and function to promote tumor cell survival. Specifically, mammary tumors induced upon carcinogen treatment of Sprague-Dawley (S-D) rats, human breast tumor cell lines, and primary human breast tumor tissue samples were found to constitutively express high levels of nuclear NF- κ B/Rel, whereas normal rat mammary glands and untransformed breast epithelial cells contained the expected low basal levels. Inhibition of this activity in breast cancer cells in culture via introduction of the specific inhibitory protein I κ B- α led to apoptosis. Inhibition of breast cancer cell growth by TGF- β 1 was shown to be mediated via decreased levels and activity of NF- κ B. More recently we have performed a time course study of induction of NF- κ B/Rel factors upon carcinogen treatment of female S-D rats, which revealed that NF- κ B/Rel activation was an early event, occurring prior to malignant transformation. Furthermore, we have shown that transformation of MCF-10F untransformed human mammary epithelial cell (HMEC) line induced by the carcinogens 7,12-dimethylbenz(*a*)anthracene (DMBA) and benzo[*a*]pyrene (BaP) transformed (lines D3-1 and BP-1, respectively) results in activation of NF- κ B/Rel subunits.

II. Body

Progress Report

Technical Objectives 1 and 2:

1. Quantitate and characterize the NF- κ B/Rel subunits induced in the DMBA and BaP transformed cell lines D3-1 and BP-1
2. Determine the kinetics of NF- κ B/Rel induction in the PAH transformation process.

These two objectives have been completed, and the work published in Kim et al., 2000 (a copy is enclosed). A brief description of the work is as follows: We examined the time course of induction of NF- κ B/Rel factors upon carcinogen treatment of female Sprague-Dawley (S-D) rats *in vivo* and in human mammary epithelial cells (HMECs) in culture. We observed that NF- κ B/Rel activation is an early event, occurring prior to malignant transformation. In S-D rats, increased NF- κ B/Rel binding was detected in nuclear extracts of mammary glands from 40% of animals 3 weeks post treatment with 15 mg/kg 7,12-dimethylbenz(a)anthracene (DMBA); this is prior to formation of tumors which normally begin to be detected after 7 to 9 weeks. In non-tumorigenic MCF-10F cells, *in vitro* malignant transformation upon treatment with either DMBA or benzo[a]pyrene (BaP) resulted in a 4- to 12-fold increase in activity of classical NF- κ B (p65/p50). NF- κ B induction was correlated with a decrease in the stability of the NF- κ B specific inhibitory protein I κ B- α . Ectopic expression of the transactivating p65 subunit of NF- κ B in MCF-10F cells induced the *c-myc* oncogene promoter, which is driven by two NF- κ B elements, and endogenous c-Myc levels. Furthermore, reduction mammaplasty-derived HMECs, immortalized following BaP exposure, showed dysregulated induction of classical NF- κ B prior to malignant transformation. Together these findings suggest that activation of NF- κ B plays an early, critical role in the carcinogen-driven transformation of mammary glands.

Technical Objective 5:

Use transgenic mice to study the contribution of c-Rel subunit expression in the development of breast neoplasia, including cross-breeding experiments with MMTV-TGF- β 1 transgenic mice to define the role of TGF- β 1 *in vivo*.

As described previously, six founder lines of transgenic mice (lines 7, 8, 14, 15, 16, & 18) were established by the Core Transgenic Facility of Boston University Medical Center. Using Southern blotting, mouse lines were shown to exhibit varying copy numbers of MMTV-c-Rel DNA. I selected the four of these lines for further study, Fo 7, 14, 15, and 16, and have been bred to homozygosity. These mice have been bred and colonies obtained as indicated in the Table 1 of the Appendix. Mammary tumors have been obtained in mice from all of the lines. Figure 1 shows the tumor appearance in one of the animals. The pathology reports for each of these tumors is included in the Appendix. These have appeared at an average of 19 months of age. Table 2 summarizes this information. Cell lines have been established in culture from several of these tumors and studies on the nature of the NF- κ B subunit expression is in progress, essentially as described in last year's progress report (see Fig. 2). In addition, the Pathology reports indicated that mice with tumors in one mammary gland showed decreased regression of the normal mammary gland after pregnancy and lactation, consistent with our previous work showing a NF- κ B protects from apoptosis.

While it was originally proposed to cross the MMTV-Rel mouse with an MMTV-TGF- β 1 mouse to see if this will delay or ablate tumor formation, the late onset of tumor appearance essentially precluded these studies. Hence I propose to alter the objective to see if crossing our mice with an MMTV-CK2 mouse can enhance the rate of tumor formation. The rationale for this change in proposed work is that we have recently shown that ectopic protein kinase CK2 (formerly known as casein kinase II) activity can increase NF- κ B expression and activity in breast cancer cell lines, and in mouse mammary tumors (Romieu-Mourez et al., 2001; Landesman-Bollag et al., 2001). Furthermore, we have shown that CK2 levels are elevated in primary human breast cancer specimens (Romieu-Mourez et al., 2001). Thus, I would test the hypothesis that the time of tumor appearance in the MMTV-Rel/CK2 bitransgenic mice will be less than in the single transgenic mice (approximate median age 19 and 23 months, respectively). I am currently in the process of performing the breeding experiments.

Technical Objectives 3 and 4:

3. Investigate the effects of TGF- β 1 on NF- κ B/Rel activity in the PAH transformed cell lines.
4. Study the cooperative effect of TGF- β 1 and chemotherapeutic drugs on cell lines.

As discussed above, the experiments on the TGF- β 1/c-Rel bitransgenic mice no longer appear feasible. Thus, I have begun examining the role of other inhibitors. Recent work by several groups have indicated that polyphenols in green tea extracts can inhibit induction of NF- κ B. Using the MMTV Her-2/neu tumor cell line, termed NF639, which we have recently shown expressed constitutive NF- κ B (Pianetti et al., 2001), we examined the effects of the most abundant polyphenol, EGCG (Kavanagh et al., 2001). EGCG reduced the level of NF- κ B binding, and inhibited growth in soft agar of NF639 cells. To determine whether there was a correlation between these two observations, I selected colonies of cells that were resistant to EGCG treatment (Fig. 3). When I analyzed NF- κ B binding in these cells, I noted that NF- κ B levels were elevated compared to the original population of cells (Fig. 4). Studies are in progress to test the mechanism of inhibition and to verify that the role of NF- κ B in transformed phenotype of these cells.

Lastly, the work described in the previous report on the ability of the RelA NF- κ B subunit to interact with the AhR has now been published (Kim et al., 2000).

Cited Literature

Kim, D.W., L. Gazourian, S.A. Quadri, R. Romieu-Mourez, D.H. Sherr, and G.E. Sonenshein. The RelA NF- κ B subunit and the Aryl Hydrocarbon Receptor (AhR) cooperate to transactivate the *c-myc* promoter. *Oncogene* 19, 5498-5506 (2000).

Pianetti, S., M. Arsura, R. Romieu-Mourez, R.J. Coffey, and G.E. Sonenshein. Her-2/neu overexpression induces NF- κ B via a PI3-kinase/Akt pathway without IKK activation that can be inhibited by the tumor suppressor PTEN. *Oncogene* 20, 1287-1299 (2001).

Landesman-Bollag, E., R. Romieu-Mourez, D.H. Song, G.E. Sonenshein, R.D. Cardiff, and D.C. Seldin. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* (in press) (2001).

Romieu-Mourez, R., E. Landesman-Bollag, D.C. Seldin, A.M. Traish, F. Mercurio, and G.E. Sonenshein. Roles of protein kinase CK2 and IKK kinases in activation of NF- κ B in breast cancer. *Cancer Res.* (in press) (2001).

Kavanagh, K.T., L.J. Hafer, D.W. Kim, K.K. Mann, D.H. Sherr, A.E. Rogers, and G.E. Sonenshein. Green tea extracts decrease carcinogen-induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. *J. Cell Biochem.* (in press) (2001).

III. Figure legends:

Fig. 1. H&E stained section of mammary tumor (adenocarcinoma) from a 19 month old, multiparous, female MMTV-c-Rel transgenic mouse.

Fig. 2. EMSA analysis was performed on nuclear extracts (5 ug) of a mammary tumor or a normal mammary gland from the same MMTV-c-Rel mouse as above, using an NF- κ B oligonucleotide as probe +/- c-Rel or p50 antibodies.

Fig. 3. Effects of EGCG on growth of NF639 cells in soft agar

Fig. 4. Colonies of NF639 cells resistant to EGCG express higher levels of NF- κ B than parental cells. A. 18 colonies survived in the presence of 40 ug/ml of EGCG were randomly picked from the soft agar plates and expanded. EMSA analysis was performed with nuclear extracts from the parental cells and with nuclear extracts from each of the clones. B. Parental cells and representative clones that have higher levels (clone11 and clone 17) or lower levels (clone 5 and clone 12) of NF- κ B than parental cells were grown in the presence or absence of 40 ug/ml of EGCG. The growth of the colonies is shown with a magnification of 40X.

Appendix

1. List of key research accomplishments (over the course of the grant):
 - NF- κ B is functionally activated in HMECs malignantly transformed by environmental carcinogens
 - In premalignant HMECs immortalized by carcinogen treatment *in vitro*, NF- κ B activity was dysregulated in quiescence.
 - Transgenic mice with targeted ectopic expression of the c-Rel subunit in the mammary gland were established, and tumor appearance is being monitored over a 2 year period.
 - MMTV-c-Rel cell lines have been established.
 - Aromatic Hydrocarbon Receptor (AhR) and RelA (p65) cooperate to transactivate the c-myc promoter in the untransformed and transformed breast epithelial cells.
2. Degrees Obtained
 - Ph.D. defense passed by the previous Principal Investigator (Dong Wook Kim). Formal degree to be awarded on June of 2001 upon completion of M.D. degree at Boston University School of Medicine.
3. Manuscripts/Presentations
 - **D.W. Kim**, M.A. Sovak, G. J. Zanieski, G. Nonet, R. Romieu-Mourez, A. W. Lau, L.J. Haefer, P. Yaswen, M. Stampfer, A.E. Rogers, J. Russo, G.E. Sonenshein. Activation of NF- κ B/Rel Occurs Early During Neoplastic Transformation of Mammary Cell. *Carcinogenesis* 21: 871-879 (2000).
 - **D. W. Kim**, L. Gazourian, S. A. Quadri, R. Romieu, D. H. Sherr, and G. E. Sonenshein. The Aromatic Hydrocarbon Receptor/Transcription Factor (AhR) and the p65 Nuclear Factor- κ B Subunit Cooperate to Transactivate the c-myc Promoter. *Oncogene* 19: 5498-5506 (2000).
 - **D. W. Kim**, M.A. Sovak, G. Zanieski, A. Lau, L. Gazourian, S. A. Quadri, R. Romieu-Mourez, G. Nonet, L.J. Haefer, P. Yaswen, M. Stampfer, J. Russo, A. E. Rogers, P. Toselli, D. Sherr, and G. E. Sonenshein. ROLE OF NF- κ B/REL, AHR AND C-MYC IN BREAST CANCER. DOD ERA OF HOPE CONFERENCE 20001, ATLANTA GA.
 - **D.W. Kim**, M.A. Sovak, M. Arsura, G. J. Zanieski, K. Kavanagh, G. Nonet, P. Yaswen, M. Stampfer, J. Russo, A.E. Rogers, G.E. Sonenshein. Early Activation of NF- κ B/Rel During Neoplastic Transformation of Mammary Cells. Russek Day Presentation, Boston University School of Medicine (2nd Prize Award).

Figure 1

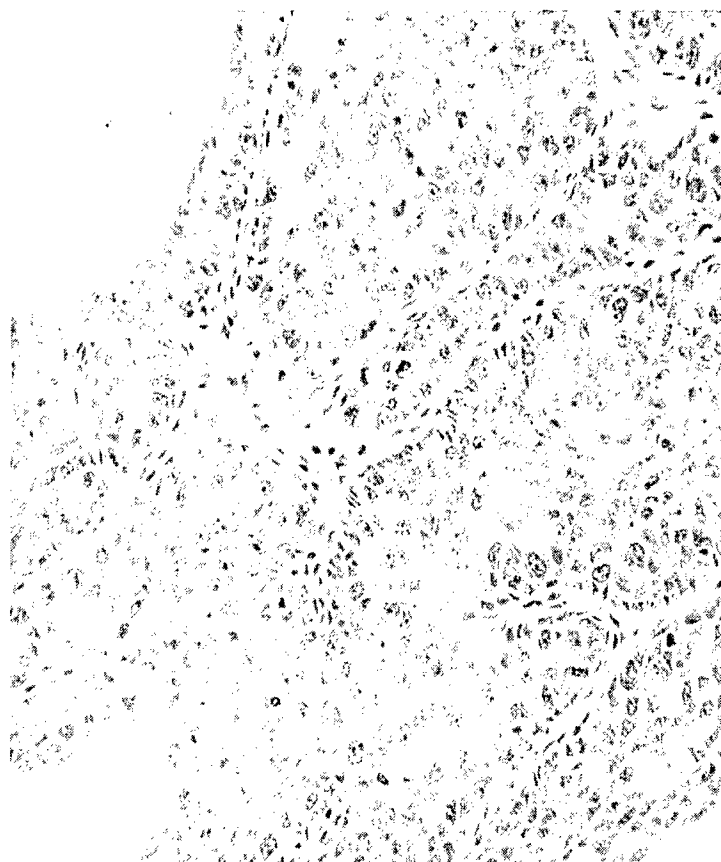


Figure 2

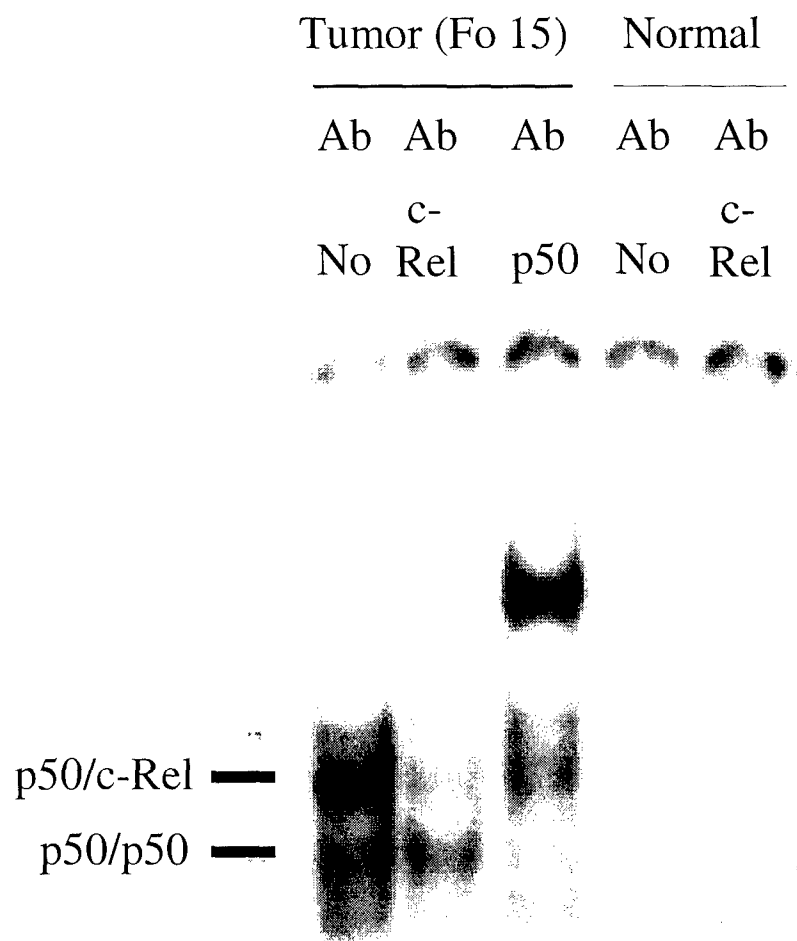


Fig. 3

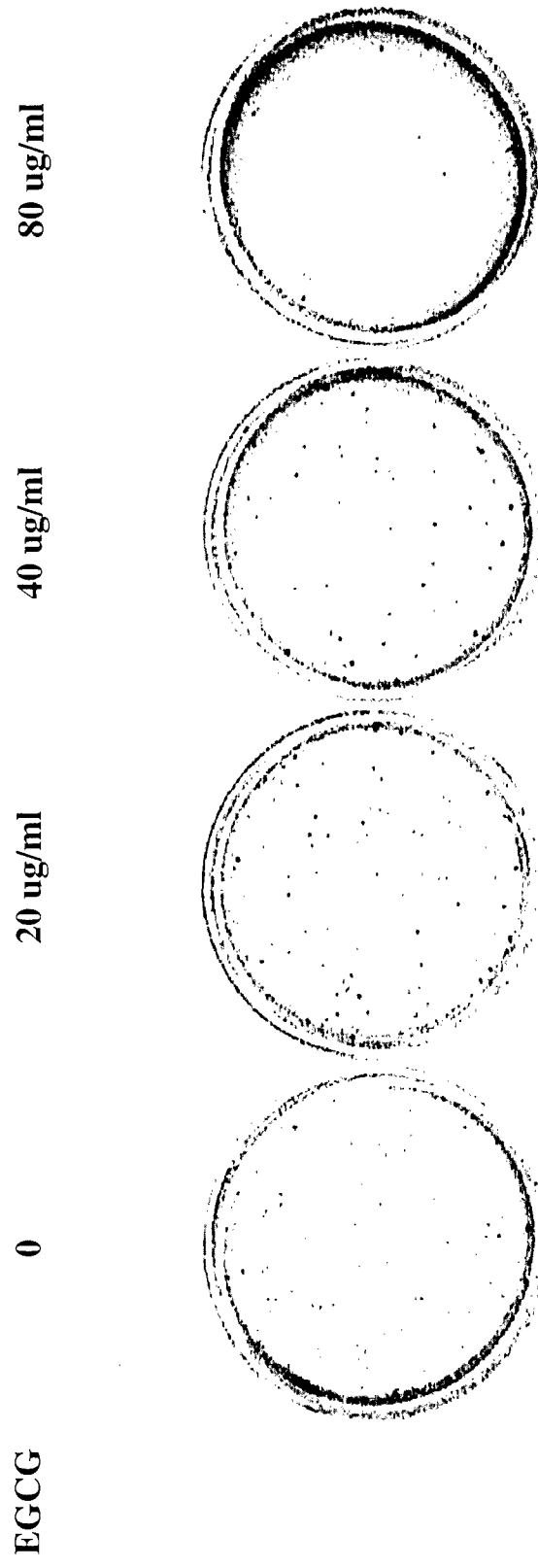


Fig.4A

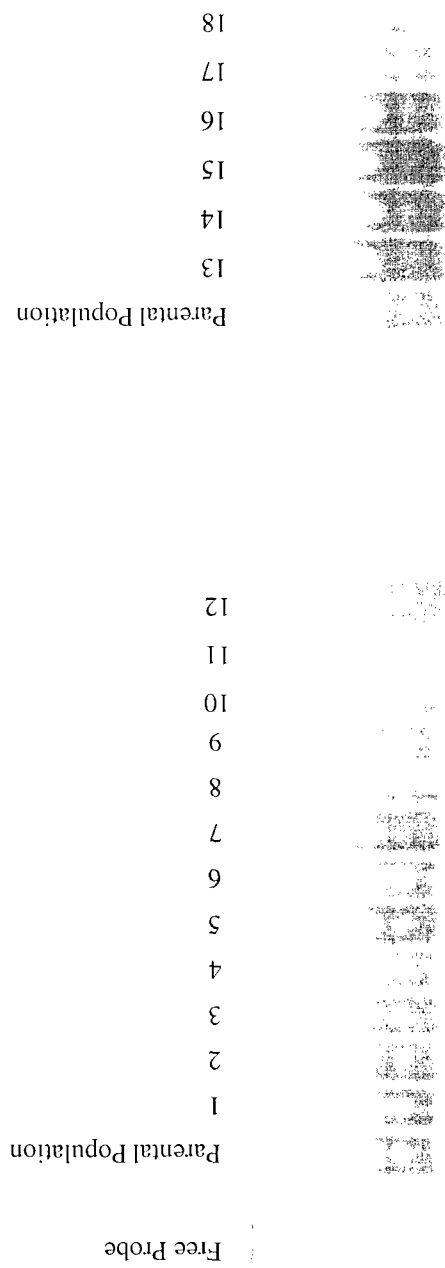


Fig 4B

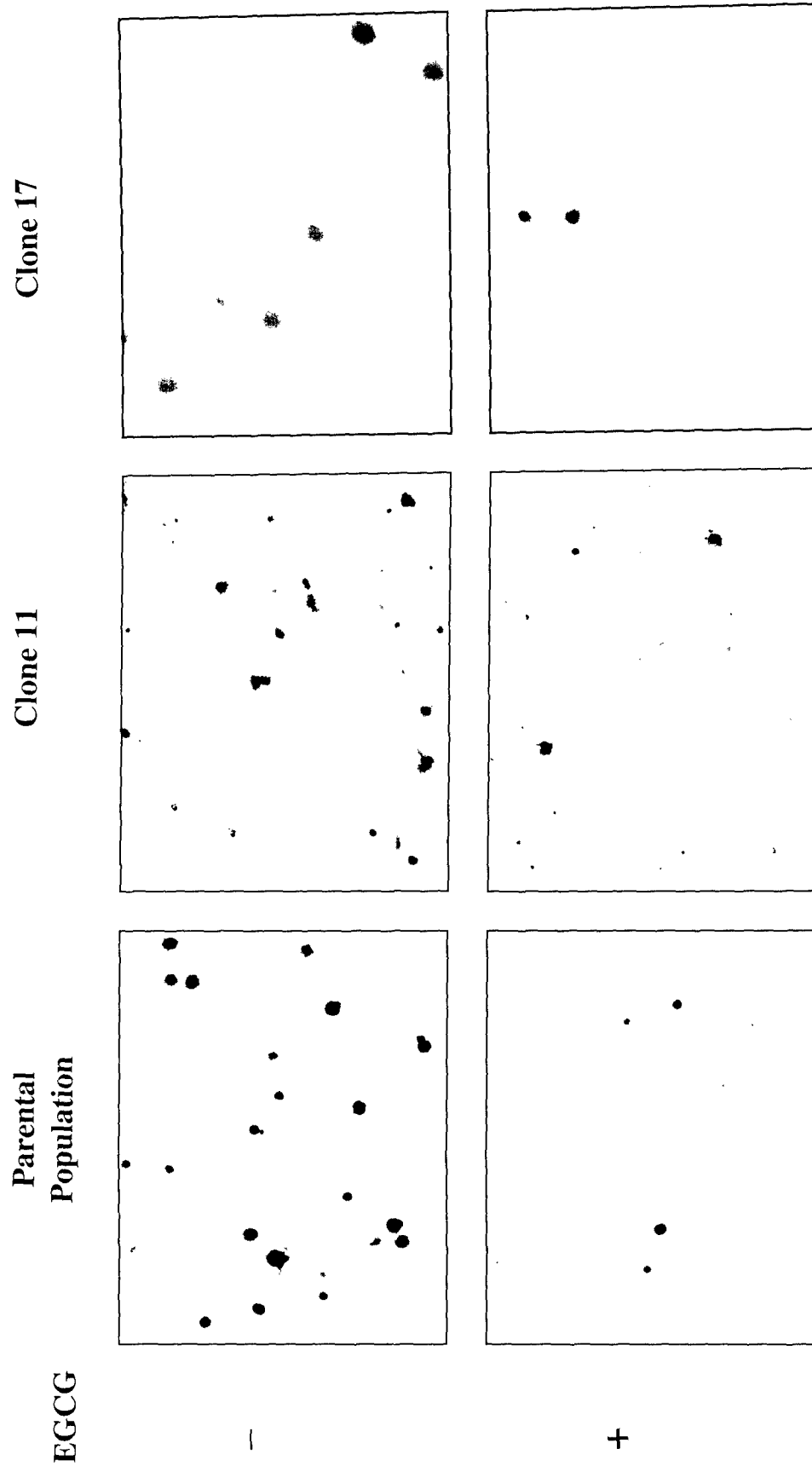


Fig. 4B (continued)

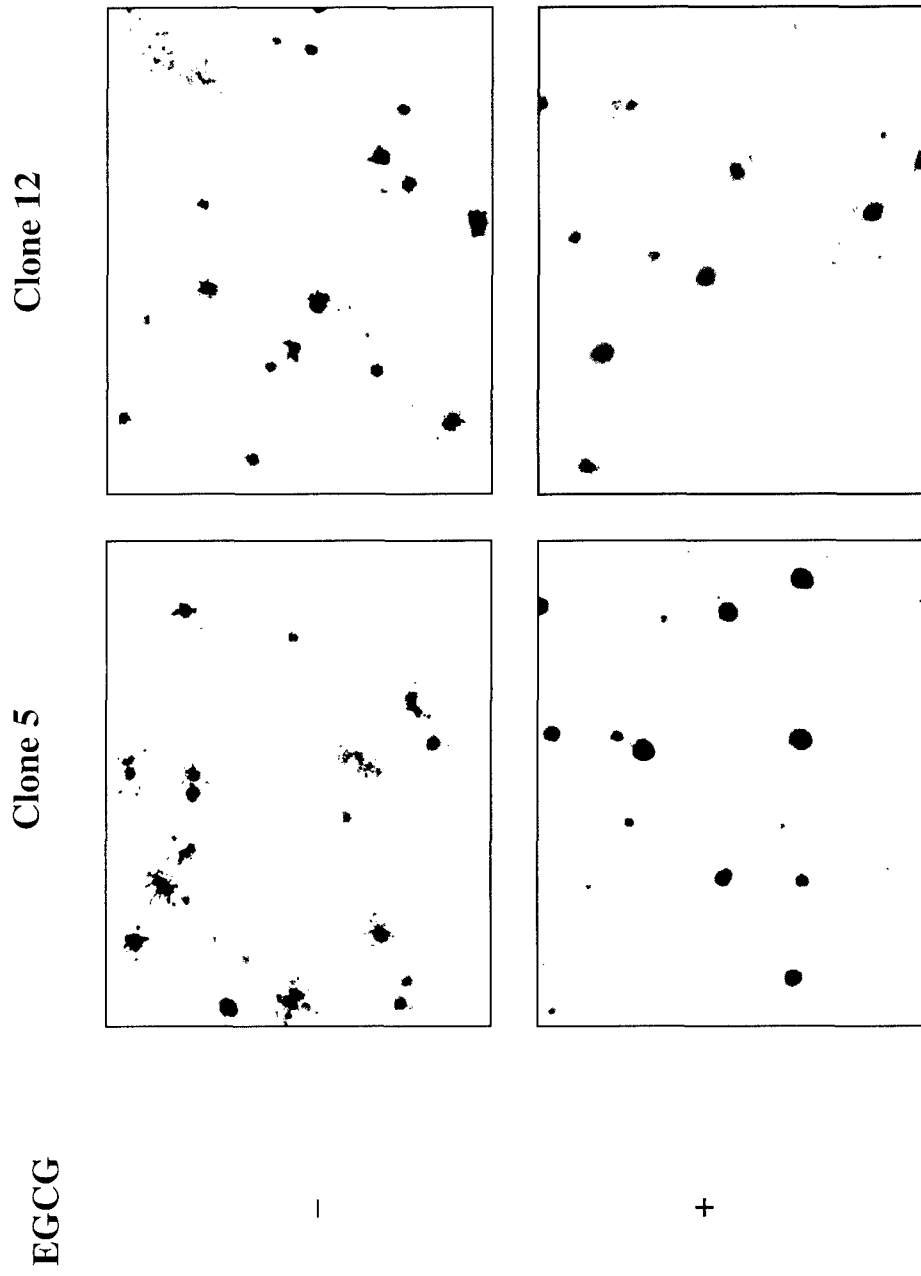


Table 1 Tumor Analysis on MMTV-c-Rel Mice *

Fo 7			
Mouse ID# ¹	Times Pregnant ²	Tumor Status ³	Maxi Age Reached To Date ⁴
3941	3	-	> 2year
4027	2	-	> 2 year
4039	5	-	> 2 year
4042	1	+	22 month
4026	4	+	22 month
4307	>1	e	17 month
5543	4	e	11 month
5423	0	e	5 month
5424	0	e	5 month

Fo 16			
Mouse ID#	Times Pregnant	Tumor Status	Maxi Age Reached To Date
3965	1	-	> 2 year
3870	>1	-	> 2 year
3872	>1	+	22.5 month
3873	>1	-	> 2 year
3875	1	+	18 month
4947	>1	e	22 month
4948	2	e	22 month
4949	1	e	22 month
4525	?	e	17.5 month
4518	1	e	17.5 month
4533	2	e	17.5 month
4527	>1	e	17.5 month
5518	1	e	12 month
5519	1	e	12 month
5567	1	e	9.5 month
5568	0	PD	7 month
5569	1	e	9.5 month
5414	0	e	4.5 month
5415	0	e	4.5 month
5416	0	e	4.5 month
5417	0	e	4.5 month
5427	0	e	3.5 month
5428	0	e	3.5 month
5429	0	e	3.5 month
5430	0	e	3.5 month
5431	0	e	3.5 month
5432	0	e	3.5 month

Table 1 (continued)

Fo 14			
Mouse ID#	Times Pregnant	Tumor Status	Maxi Age Reached To Date
N.A. 5	>1	+	16.5 month
3814	3	+	26 month
3815	2	-	> 2 year
3813	2	-	> 2 year
3981	3	-	> 2 year
3982	3	-	> 2 year
3983	3	+	20.5 month
3996	3	+	23 month
4936	6	e	22 month
4540	2	e	17 month
4970	>1	e	21 month
5532	4	e	12 month
5530	4	e	12 month
5592	0	e	6.5 month
5593	1	e	6.5 month
5594	1	e	6.5 month
5595	0	e	6.5 month
5596	0	e	6.5 month
5597	2	e	6.5 month
5598	0	e	6.5 month
5599	0	PD	4 month
5600	0	e	6.5 month
5401	0	e	6.5 month
5402	0	e	6.5 month
5403	0	e	6.5 month
5404	2	e	6.5 month
5405	0	PD	4 month
5437	1	e	3 month
5438	1	e	3 month
5439	1	e	3 month
5440	0	e	1.5 month
5441	0	e	1.5 month
5442	0	e	1.5 month
5443	0	e	1.5 month
5444	0	e	1.5 month
5445	0	e	1.5 month
5446	0	e	1.5 month

Table 1 (continued)

Fo 15			
Mouse ID#	Times Pregnant	Tumor Status	Maxi Age Reached To Date
127	>1	+	19 month
3759	6	-	2 year
4005	5	-	> 2 year
4922	>1	e	22 month
4923	>1	PD	18.5 month
4010	2	PD	<22 month
4513	>1	e	17.5 month
4515	>1	PD	<13 month
5503	5	e	12 month
5307	5	e	12 month
5571	0	e	9 month
5572	0	e	9 month
5574	0	e	9 month
5575	0	e	9 month
5409	0	e	5.5 month
5410	0	e	5.5 month
5411	0	e	5.5 month

* 24 month endpoint. Information is given for each founder line separately.

1. Mouse ID number in the colony. The presence of the transgene is confirmed by Southern blot analysis. Only the female mice are listed.
2. Times of pregnancy during the life time to date.
3. Tumor status; -, no tumor detected to date; +, mammary tumor; PD, premature death; e, experiment in progress.
4. Maxi age reached to date, individual mouse age to date listed. For mice developed tumor or prematurely dead, the ending time of their age is the time when they are sacrificed or dead.
5. The ID# of this mouse is not available because of the loss of its ear tag. The presence of the transgene was reconfirmed by Southern blot analysis of the tail DNA.

Table 2. Mammary Tumor Formation In The MMTV-c-Rel Transgenic Mice

Founder Line	Mouse ID#	Mouse Age When Tumor Appeared (month)
7	4026	22
7	4042	22
14	3814	26
14	3983	20.5
14	3996	24
14	N.A.	16.5
15	127	19
16	3872	22.5

* The ID# of this mouse is not available because of the loss of its ear tag.
The presence of the transgene was reconfirmed by Southern blot analysis
of the tail DNA.

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory BOSTON UNIV Investigator(s): SONENSHEIN ID Number: FO16 3872
22.5 months
Submitter(s): SHIN Date In: 8/28/00 Animal Strain/Number: FVB /
Experimental: TRANSGENIC Control: Carcinogen:
Virus: Age 0 W Sex: F Sample Number: FO16 3872

Promoter 1: MMTV	Promoter 2:	Promoter 3:
Transgene 1: CREL	Transgene 2:	Transgene 3:
Genotype 1:	Genotype 2:	Genotype 3:

Date in which the tumor first noted: 8/17/00 DOB: 10/2/98 DOD:
Mouse: Yes Other Species: Pregnancies: 2
Fixative: OMNIFIX Post-fix: Days of Fixation:
Processing: 0 ASAP: 0 Collected: 0 Fixed: 0 Frozen: 0

Gross description/ experimental notes:

Small growth found on the back of her neck. It was well solid by the time of dissection. It is suspected to be tumor which is included with other organs such as liver, lung, heart, spleen, adrenal, kidney.

U.C. Davis Slide Number: 00-0865BU

Microscopic Description:

TG00-0865Bu (3872)(SONNENSHEIN/LAU)

Three H&E stained slide with uterus and stroma from a multiparous mtv-Rel-1 female mouse are examined. Slides 1 and 2 have samples of normal spleen, liver, adrenal and kidney. Slide 3Bu has a large squamous cell carcinoma with infiltrating borders and a cystic center that is filled with laminar keratin. The lung is without metastases.

COMMENT: This appears to be a pure squamous cell carcinoma. This type of tumor is rare in mice.

Diagnosis: MAMMARY SQUAMOUS CELL CARCINOMA WITHOUT PULMONARY METASTASES.

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV. Investigator(s): LAU ID Number: F07 4026
22 months
Submitter(s): SONSENSHIEN Date In: 10/6/00 Animal Strain/Number: FVB /
Experimental: TRANSGENIC Control: Carcinogen:
Virus: Age: 0 W Sex: F Sample Number: 4026

Promoter 1: MMTV	Promoter 2:	Promoter 3:
Transgene 1: CREL	Transgene 2:	Transgene 3:
Genotype 1:	Genotype 2:	Genotype 3:

Date in which the tumor first noted: 9/13/00 DOB: 11/23/98 DOD: 9/28/00

Mouse: Yes Other Species: Pregnancies:
Fixative: OMNIFIX Post-fix: Days of Fixation:
Processing: 0 ASAP: 0 Collected: 0 Fixed: 0 Frozen: 0

Gross description/ experimental notes:

Large growth found on left side of mouse near L4 and L5. The growth was solid with partial dark red lump on the side which was attached to the left rear leg/thigh. Included samples are suspected tumor, tissues from L1,2,3, liver, lung, heart, spleen, adrenal and kidney. 3 cassettes.

U.C. Davis Slide Number: 00-0930BU

Microscopic Description:

TG00-0930Bu (4026)(SONENSHEIN/LAU)

Three H&E stained slides from a MMTV-Rel female mouse are examined. Slide 1 contains three samples of mammary fat pad. One of the three samples has a large expansile mammary tumor. The tumor is cystic with the center occupied by a pink keratinaceous debris. The edges of the tumor have hyperchromatic basaloid cells that tend to differentiate towards a stratified squamous epithelium. The rest of the mammary gland has numerous residual lobules with extensive fibrosis and inflammation. Three foci stand out from the background with irregular glands containing large pleomorphic, hyperchromatic cells. Slide 2 contains kidney, liver and heart. The spleen on Slide 2 has an accentuation of the white pulp with no evidence of germinal centers. Slide 3 contains normal liver.

COMMENT: The pattern observed in the spleen suggests a lymphocytic lymphoma.

Diagnosis: SQUAMOUS CELL CARCINOMA, DIFFUSE HYPERPLASIA AND FOCAL ATYPICAL NODULES, MAMMARY GLAND. ATYPICAL LYMPHOID INFILTRATE, SPLEEN.

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory BOSTON UNIV Investigator(s): SHIN ID Number: Fo 7 4042
 Submitter(s): SONENSHEIN Date In: Animal Strain/Number: Fo 7 / 22 months
 Experimental: Transgenic Control: — Carcinogen: —
 Virus: — Age 5E+04 W Sex: f Sample Number:

Promoter 1: MMTV-LTR	Promoter 2: NONE	Promoter 3: NONE
Transgene 1: cREL	Transgene 2: —	Transgene 3: —
Genotype 1: UNKNOWN	Genotype 2: NONE	Genotype 3: NONE

Date in which the tumor first noted: 12/1/00¹ DOB: 2/4/99 DOD: 1/1/00¹
 Mouse: No Other Species: Pregnancies: 4
 Fixative: Omnifix Post-fix: Alcohol Days of Fixation: —
 Processing: -1 ASAP: 0 Collected: -1 Fixed: -1 Frozen: 0

Gross description/ experimental notes:

LARGE GROWTH FOUND ON LEFT SIDE OF ANIMAL NEAR L4,5. UPON REMOVAL OF GROWTH, YELLOWISH FLUID WITH WHITE SOLIDS WAS DISPENSED. IT STILL CONTAINED LOW AMOUNT OF VASCULARIZATION. WHEN ANIMAL WAS OPENED UP, A BUBBLE FILLED WITH YELLOW FLUID WAS DISCOVERED NEAR KIDNEY. IT WAS DISPENSED WHEN ATTEMPTED TO REMOVE IT. INCLUDED PIECES OF SUSPECTED TUMOR AND NON TUMOR MAMMARY GLAND (R4,5). ALSO SOME TISSUES FROM LIVER, LUNG, HEART, SPLEEN AND KIDNEY. 2 CASSETTES.

U.C. Davis Slide Number: 01-0013BU

Microscopic Description:

TG01-0013H(4042)(SONENSHEIN/SHEN)

Two H&E stained slides from a 10 month old multiparous cRel female mouse are examined. Slide 1Bu contains multiple tissue samples. A partially necrotic tumor mass is present that has a mixture of poorly formed glands and squamous epithelium with extensive laminar keratin. The kidneys have mixed lymphoplasmacytic infiltrates that are perivascular. The liver is normal. The red pulp of the spleen is partially replaced by a ill-defined population of large lymphoid cells. Slide 2Bu contains mammary gland that is 70% filled with partially regressed mammary gland that retains extensive LA development. One focus of atypical acinar cells with fibrosis and interstitial inflammation is present. The myocardium and lungs are normal.

COMMENT: Please use our new request forms found in hard copy and electronic format at URL: <http://ccm.ucdavis.edu/tgmouse/>. The spleen could have an early form of leukemia/lymphoma but hematologic data such as blood smears would be needed to confirm the diagnosis. The tumor sample does not contain adjacent host tissue so the identification of its origin is impossible.

Diagnosis: ADENOSQUAMOUS CARCINOMA, SITE NOT SPECIFIED. LYMPHOID HYPERPLASIA, SPLEEN.

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV Investigator(s): SONENSHEIN ID Number: FO14 3814
Submitter(s): SHIN Date In: 3/5/01 Animal Strain/Number: FVB / *> 2 years*
Experimental: Transgenic Control: — Carcinogen: —
Virus: — Age 129 W Sex: f Sample Number:

Promoter 1: MMTV-LTR	Promoter 2: NONE	Promoter 3: NONE
Transgene 1: c-REL	Transgene 2: —	Transgene 3: —
Genotype 1: +/+	Genotype 2: NONE	Genotype 3: NONE

Date in which the tumor first noted: 2/6/01 DOB: 8/27/98 DOD: 2/16/01
Mouse: Yes Other Species: Pregnancies: 3
Fixative: Omnifix Post-fix: Alcohol Days of Fixation: —
Processing: -1 ASAP: 0 Collected: -1 Fixed: -1 Frozen: 0

Gross description/ experimental notes:

Upon dissection, suspected tumor was exposed around R4,5 of mammary glands. It was partially filled with yellowish liquids. During further dissection, two yellow, liquid filled spheres were found near liver and kidney. I have included R1,2 mammary glands as well as liver, kidney, lung, heart, spleen, and suspect tumor (R4,5). 3 cassettes.

U.C. Davis Slide Number: 01-0079BU

Microscopic Description:

TG01-0079(3814)(SHIN/SONENSHEIN)

Three H&E stained slides from 129 week old cRel female are examined. Slide 1Bu has two samples of large partially necrotic, partially keratinized masses in the mammary gland. The bulk of the tissue is composed of glandular epithelium with small hyperchromatic nuclei and scanty cytoplasm. The adjacent gland has several dilated glands that are lined by a micropapillary atypical epithelium. The liver and kidney is normal. Slide 2 has normal liver, spleen and heart. The epicardial surface has a round cell infiltrate. Slide 3 has lung that also has an intense infiltrate of lymphocytes.

COMMENT: The pattern suggests a leukemic infiltrate.

Diagnosis: ADENOSQUAMOUS MAMMARY CARCINOMAS. ATYPICAL MAMMARY PAPILLARY
HYPERPLASIA. LYMPHOCYTIC PLEURITIES AND EPICARDITIS.

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV Investigator(s): SELDIN/SONENSHEIN ID Number: FVB#127
Animal Strain, Number: / Genotype: mmtv-c-Re1 Sample Number: 6.15
Sex: F DOB: 6/2/98 DOD: Mouse: Yes Other Speci: 19 month
Date in which the tumor (or other pathology) is first noted: 1/12/00 Pregnancies:

Tumor Passaged
<input type="radio"/> In vivo
<input type="radio"/> In Culture

Fixative
<input checked="" type="radio"/> Omnifix
<input type="radio"/> Formalin

Processed
<input checked="" type="radio"/> Routinely
<input checked="" type="radio"/> ASAP
Other:

Gross description/ experimental notes:

Two H&E slides submitted.

U.C. Davis Slide Number: 00-0120BU

Microscopic Description:

TG00-0120Bu (9592)(SONENSHEIN/LAU)

Two H&E stained slides with samples from a 9 month old multiparous cRel female mouse are examined. Slide 1Bu has normal liver, kidney, spleen and lung. Slide 2Bu has lung, ovary and mammary glands. The mammary glands are partially regressed. One has a large multicystic tumor that is composed of small glands and large cysts. The glands have large cells with large pleomorphic nuclei and scanty cytoplasm. The nuclei have a open chromatin pattern with very large nucleoli. The mitotic rate is high. The adjacent and contralateral mammary gland have focal areas of atypical glands that stand out from the background. The lung has three very large papillary neoplasms that compress the surrounding lung and create a lipoid pneumonia. The ovary does not have any eggs or Graffian follicles.

COMMENT: The mammary tumor could be similar to the standard Type C described by Thelma Dunn. However, the cytology is unusual and suggests that the transgene has had a primary effect. The masses in the lungs are bronchial adenocarcinomas that are frequently found in FVB mice. They are not metastases.

Diagnosis: MAMMARY ADENOCARCINOMA AND ATYPICAL HYPERPLASIA. PAPILLARY BRONCHIAL ADENOCARCINOMAS.

Pathologist: NIETO/CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory BOSTON UNIV

Investigator(s): SO/LAU

ID Number: Fo16 3875

Animal Strain, Number: /

Genotype: mmtv-c-Re1

Sample Number:

18 months

Sex: F

DOB: 10/2/98

DOD:

Mouse: Yes

Other Speci:

Date in which the tumor (or other pathology) is first noted:

Pregnancies:

Tumor Passaged

- ☐ In vivo
☒ In Culture

Fixative

- ☒ Omnifix
☐ Formalin

Processed

- ☒ Routinely
☒ ASAP

Other:

Gross description/ experimental notes:

One paraffin block submitted. Upon dissection, a fairly large growth (1-1.5cm in diameter) was found around the mammary gland L2. In addition, the spleen seemed to be enlarged as well. Other than these observations, the mouse seemed normal. Samples included for pathology: suspected tumor (L2), normal mammary gland (L4), salivary gland, thymus, lung, heart, liver, kidney, and spleen (enlarged).

U.C. Davis Slide Number:

00-0266BU

Microscopic Description:

TG00-0266Bu (3875)(SONENSHEIN/LAU)

One H&E stained slide with various organs from an eighteen month old Fo16p (mmTV/c-rel) female is examined. A large tumor mass is present adjacent to mammary tissue. The mass is surrounded by a dense connective tissue stroma. The tumor is composed of small clusters of neoplastic cells in a dense stroma. The cells have very large, pleomorphic, hyperchromatic nuclei with very large, central nucleoli. The cytoplasm is abundant and pink. Some nests form abortive, ill-defined glands. The mitotic rate is high. The adjacent mammary tissue is sparse but has some atypical cells. The other mammary gland has residual alveoli but without atypical cells. The thymus is atrophic. The spleen has a prominent white pulp but without atypia. The lungs have no metastases.

COMMENT: This is a very unusual tumor in the G.E.M. mammary gland. In the future, please provide more of the normal host tissue adjacent to the tumor for analysis.

Diagnosis: POORLY DIFFERENTIATED LARGE CELL MAMMARY ADENOCARCINOMA.**Pathologist:** CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV. Investigator(s): So/LAU ID Number: Fo14-3983
Animal Strain, Number: FVB / 3983 Genotype: MMTV-crel Sample Number: 20-5 months
Sex: F DOB: 8/12/98 DOD: Mouse: No Other Speci:
Date in which the tumor (or other pathology) is first noted: 4/25/00 Pregnancies:

Tumor Passaged

- ☐ In vivo
☐ In Culture

Fixative

- ☒ Omnifix
☐ Formalin

Processed

- ☒ Routinely
☒ ASAP
Other:

Gross description/ experimental notes:

8 SAMPLES IN BAG. Upon dissection, a large growth (2cm in diameter) was observed around mammary gland RR4+5. Once the growth was cut into, a white fluid was released. Other than these observations, the mouse seemed normal. Samples submitted, suspect tumor, piece of surrounding normal, heart, lung, spleen, kidney, adrenal gland, liver and L4 normal mammary tissue.

U.C. Davis Slide Number: 00-0451BU

Microscopic Description:

TG00-0450Bu (3983)(SONNENSHEIN/LAU)

One H&E stained slide with multiple samples of organs from a parous c-Rel female mouse is examined. The mammary gland shows partial regression with the retention of many lobules. The mammary gland has a small squamous nodule and a large tumor that is composed of a combination of glandular and squamous epithelium. The kidney has extensive interstitial fibrosis and chronic inflammation. The lung has two small papillary tumors. The smaller mass is within a bronchus. The liver, heart and spleen are within normal limits.

COMMENT: The mammary tumor is of the type that is occasionally found in wild type FVB mice.

agnosis: MAMMARY ADENOSQUAMOUS CARCINOMA (KERATOACANTHOMA), SQUAMOUS NODULE
AND INCOMPLETE INVOLUTION, MAMMARY GLAND. PAPILLARY BRONCHIAL ADENOMAS,
LUNG CHRONIC INTERSTITIAL NEPHRITIS

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV

Investigator(s): SELDIN/SONSENHEI ID Number:

Animal Strain, Number: /

Genotype: mmtv

Sample Number:

Sex: F

DOB: 9/7/98

DOD:

Mouse: Yes

Other Speci:

Date in which the tumor (or other pathology) is first noted: 1/26/00

Pregnancies:

Tumor Passaged

- ☐ In vivo
☒ In Culture

Fixative

- ☒ Omnifix
☐ Formalin

Processed

- ☒ Routinely
☒ ASAP
Other:

Gross description/ experimental notes:

Upon dissection, a large, well vasculized growth was found around the mammary gland R4. Other than this growth, the moused seemed normal and well. Samples included for pathology: suspected tumor (R4) and piece of adjacent normal mammary tissue, liver, spleen, kidney, salivary gland, heart, lung, lymphnode.

U.C. Davis Slide Number: 00-0164BU

Microscopic Description:

TG00-0164Bu (NO ID NUMBER)(SELDIN/SONENHEIM)

One H&E stained slide from a female labeled MMTV is examined. The spleen shows a myeloid hyperplasia. The mammary fat pad has an invasive neoplasm composed of elongate cells with giant pleomorphic hyperchromatic nuclei. The cytoplasm is abundant and contains visible cross striations. Many of the cells at the periphery are smaller and have less cytoplasm. Mitotic figures are sparse. The other organs are within normal limits.

COMMENT: This is a rare tumor.

Diagnosis: RHABDOMYOSARCOMA, MAMMARY FAT PAD. MYELOID HYPERPLASIA AND IMMATURITY, SPLEEN.

Pathologist: CARDIFF

University of California Transgenic Laboratory

Pathology Report

Submitting Laboratory: BOSTON UNIV Investigator(s): SONENSHEIN ID Number: FO14 3996
Submitter(s): SHIN Date In: 8/28/00 Animal Strain/Number: FVB / *24 months*
Experimental: TRANSGENIC Control: Carcinogen:
Virus: Age 0 W Sex: F Sample Number: FO14 3996

Promoter 1: MMTV	Promoter 2:	Promoter 3:
Transgene 1: CREL	Transgene 2:	Transgene 3:
Genotype 1:	Genotype 2:	Genotype 3:

Date in which the tumor first noted: 8/9/00 DOB: 9/17/98 DOD:
Mouse: Yes Other Species: Pregnancies: 3
Fixative: OMNIFIX Post-fix: Days of Fixation:
Processing: 0 ASAP: -1 Collected: 0 Fixed: 0 Frozen: 0

Gross description/ experimental notes:

Large growth found on right side of animal near R1 & 2 and possibly R3. Low amount of vascularization and part of growth appeared cystic and filled with clear fluid. A separate growth, somewhat vascularized found close to the thyroid area, but clearly attached to muscle tissue of upper right foreleg. Included pieces of both suspected tumors with as much surrounding tissue as possible. Also some tissue from L 2,3 liver, lung, heart, spleen adrenal, kidney. 6 cassettes.

U.C. Davis Slide Number: 00-0864BU

Microscopic Description:

TG00-0864Bu (3996)(SONNENSHEIN/LAU)

Six H&E stained slides with uterus and stroma from a multiparous mtv-Rel-1 female mouse are examined. Slide 1Bu contains normal liver. Slide 2Bu contains two large mammary tumors that are composed of large and somewhat cystic nodules of tumor cells. The cells are arranged in a mixture of papillary and acinar patterns. The cells have hyperchromatic nuclei and abundant cytoplasm with some clear vacuoles (indicating secretions). The adjacent mammary gland is only partially regressed. The adjacent skeletal muscle has an intense inflammation and fibrosis. Slide 3Bu contains normal heart and spleen. The spleen has a relative excess of lymphoid pulp. Slide 4Bu contains normal salivary gland and four fragments of mammary gland. One fragment has ectatic ducts and residual lobules. Another has a round mass that is filled with lactational alveoli. The center of this mass contains a squamous nodule. The other two masses are mammary adenocarcinomas with papillary patterns. Slide 5Bu contains adrenals and kidney. The kidney has scattered interstitial infiltrates of plasma cells and lymphocytes. The kidneys also have small cortical retention cysts. Slide 6 contains more liver and a sample of lung. The lung has 5 microscopic foci of metastatic adenocarcinoma that have grown through the vessels and into the parenchyma. Multiple tumor emboli are present.

COMMENT: Thank you for the detailed clinical description. It is very helpful.

Diagnosis: MAMMARY ADENOCARCINOMAS (4) WITH PULMONARY METASTASES.

Pathologist: CARDIFF



The RelA NF- κ B subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-*myc* promoter in mammary cells

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NF- κ B/Rel transcription factors regulate many genes involved in control of cellular proliferation, neoplastic transformation, and apoptosis, including the c-*myc* oncogene. Recently, we have observed that levels of NF- κ B and aryl hydrocarbon receptor (AhR), which mediates malignant transformation by environmental carcinogens, are highly elevated and appear constitutively active in breast cancer cells. Rel factors have been found to functionally interact with other transcription factors. Here we demonstrate a physical and functional association between the RelA subunit of NF- κ B and AhR resulting in the activation of c-*myc* gene transcription in breast cancer cells. RelA and AhR proteins were co-immunoprecipitated from cytoplasmic and nuclear extracts of non-malignant MCF-10F breast epithelial and malignant Hs578T breast cancer cells. In transient co-transfection, RelA and AhR gene products demonstrated cooperation in transactivation of the c-*myc* promoter, which was dependent on the NF- κ B elements, and in induction of endogenous c-Myc protein levels. A novel AhR/RelA-containing NF- κ B element binding complex was identified by electrophoretic mobility shift analysis of nuclear extracts from RelA and AhR co-transfected Hs578T cells. Thus, the RelA and AhR proteins functionally cooperate to bind to NF- κ B elements and induce c-*myc* gene expression. These findings suggest a novel signaling mechanism whereby the Ah receptor can stimulate proliferation and tumorigenesis of mammary cells. *Oncogene* (2000) 19, 5498–5506.

Keywords: NF- κ B; RelA; AhR; c-*myc* oncogene; breast cancer

Introduction

NF- κ B/Rel is a family of dimeric transcription factors characterized by the presence of a Rel homology region (RHR) of about 300 amino acids in length, which controls multiple functions including dimerization, DNA binding, and nuclear localization. Classical NF- κ B is a heterodimer composed of p65 (or RelA) and p50 (or NF κ B1) subunits (Grimm and Baeuerle, 1993).

The RelA subunit has potent transactivation potential, while the p50 subunit has only modest transactivation ability *in vivo* (Grimm and Baeuerle, 1993; Ballard *et al.*, 1992; La Rosa *et al.*, 1994). Many genes are regulated by NF- κ B (Grilli *et al.*, 1991; Grimm and Baeuerle, 1993). For example, we demonstrated that the c-*myc* oncogene is potently transactivated by NF- κ B/Rel factors (La Rosa *et al.*, 1994). In most cells, other than B lymphocytes, NF- κ B/Rel proteins are sequestered in the cytoplasm bound to one of the specific inhibitory proteins termed I κ Bs of which I κ B- α is the paradigm. A variety of agents can induce NF- κ B/Rel, including oxidative stress (Grimm and Baeuerle, 1993; Verma *et al.*, 1995). Activation of NF- κ B involves phosphorylation and degradation of I κ B, which allows for translocation of an active NF- κ B complex into the nucleus where it can bind to NF- κ B responsive elements (Verma *et al.*, 1995). However, we recently demonstrated that breast cancer cell lines and primary breast cancer specimens are typified by aberrant constitutive activation of NF- κ B (Sovak *et al.*, 1997).

We have postulated that one mechanism leading to constitutive NF- κ B activation may be oxidative stress induced by activation of cytochrome P450 enzymes, some of which are regulated by the aryl hydrocarbon receptor (AhR) (Nebert *et al.*, 1990, 1991). The AhR is a cytosolic protein complexed with heat shock protein (Hsp90) and an immunophilin-like molecule, ARA-9/XAP-2/AIP (Carver and Bradfield, 1997; Jain *et al.*, 1994; Perdew and Bradfield, 1996; Ma and Whitlock, 1996; Meyer *et al.*, 1998), and c-Src (Enan and Matsumura, 1996). Acute activation can occur with multiple agents, including classes of carcinogenic environmental chemicals (e.g. dioxins, polycyclic aromatic hydrocarbons (PAH), and planar polychlorinated biphenyls (PCBs)). Upon activation, the receptor translocates to the nucleus, binds specific response elements (XREs), and induces transcription of a number of genes, including those encoding the P450 enzymes CYP1A1, CYP1A2, and CYP1B1. As predicted from the working model, we have recently found high levels of constitutively active AhR in PAH-induced rat mammary tumors (Trombino *et al.*, 2000), that coincide with constitutively active NF- κ B (Sovak *et al.*, 1997).

The recent work of Tian *et al.* (1999) suggests a second pathway linking AhR and NF- κ B activities. In particular, they demonstrated a physical association between the AhR and the RelA subunit of NF- κ B in murine hepatoma cells, and transcriptional down-

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³These authors contributed equally to this manuscript

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regulation by AhR-NF- κ B subunit complexes of an NF- κ B element driven construct. To test for this additional regulatory mechanism in human breast epithelial and tumor cell lines, the potential for AhR and NF- κ B subunits to associate and regulate an NF- κ B-regulated gene promoter, i.e. *c-myc*, was examined. We report that the RelA and AhR proteins co-precipitate in untransfected or *RelA*- and *AhR*-transfected human mammary epithelial cell (HMEC) lines. In contrast to the previous report by Tian and coworkers (1999), however, we find RelA and AhR cooperate to positively transactivate the *c-myc* gene, apparently via direct binding to NF- κ B elements. These findings suggest a new mechanism whereby aberrant constitutive NF- κ B/AhR expression can promote activation of the *c-myc* gene and thereby proliferation and neoplastic transformation.

Results

RelA and AhR are associated in HMECs

To assess the association of the RelA subunit of NF- κ B with AhR in breast epithelial cells, co-immunoprecipitation studies were performed. Total cell, nuclear, and cytoplasmic extracts were prepared from malignant Hs578T breast cancer cells, which have been found to express both RelA (Sovak *et al.*, 1997), and AhR proteins. Samples of the nuclear (50 μ g) and cytoplasmic (100 μ g) fractions were treated with either a goat antibody against AhR (Figure 1a, lanes 2,3) or a goat IgG fraction, as control (Figure 1a, lanes 4,5). Immune complexes were isolated using protein A-Sepharose and subjected to electrophoresis, along with a sample of total cell lysate (Figure 1a, lane 1). The resulting immunoblot was probed with a rabbit polyclonal antibody for expression of the 65 kDa RelA subunit. In the total cell lysate, RelA-specific antibody recognized a protein of the expected molecular weight (65 kDa). The AhR antibody co-precipitated RelA protein from either cytoplasmic or nuclear extracts (Figure 1a), with somewhat greater amounts seen with the cytoplasmic sample. In contrast, the control goat IgG failed to co-precipitate detectable levels of RelA protein. To confirm this association we performed the inverse experiment of immunoprecipitating nuclear (100 μ g protein) or cytoplasmic (200 μ g protein) extracts with a rabbit antibody against RelA and then immunoblotting for AhR using a goat antibody. The RelA antibody co-precipitated AhR protein from either cytoplasmic or nuclear extracts (Figure 1b). In contrast, the control rabbit IgG failed to co-precipitate detectable levels of AhR protein. As seen above, somewhat greater amounts of complexes were detected in the cytoplasm. These findings suggest that endogenous AhR is associated with RelA in both the nucleus and the cytoplasm of Hs578T cells; although, the majority of the complexes, as judged from this and two duplicate experiments (0.80 ± 0.07), are present in the cytoplasm.

We next sought to assess the ability of RelA to associate with the AhR in non-malignant MCF-10F HMECs. To this end, MCF-10F cells were transfected with a vector expressing the *T7-pcDNA3-AhR* vector encoding T7-tagged AhR. Alternatively, cells were

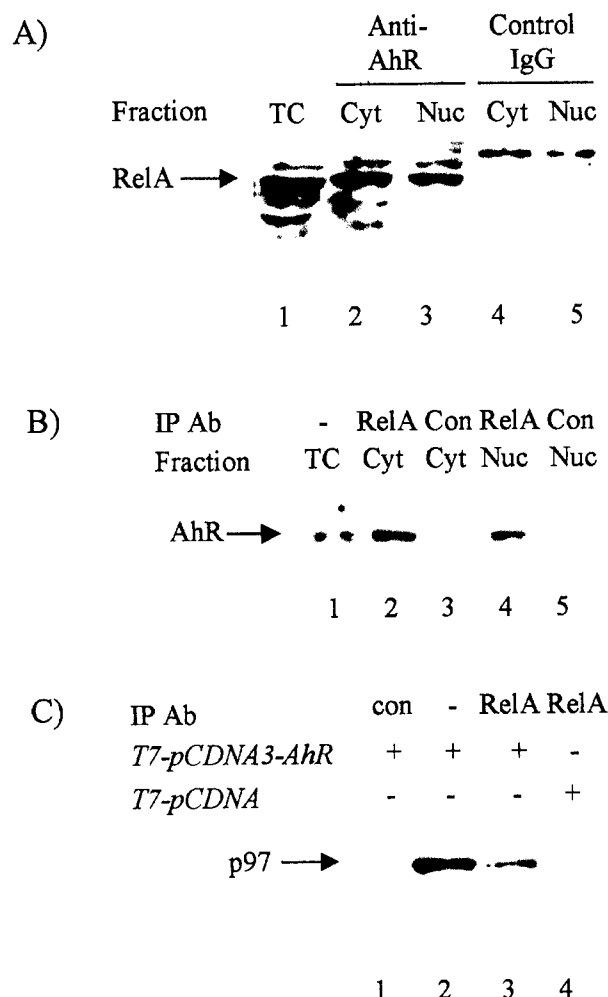


Figure 1 AhR and RelA are associated in Hs578T and MCF-10F cells. (a) Cytosolic (100 μ g from 1 mg total) or nuclear (50 μ g from 128 μ g total) proteins from Hs578T cells were immunoprecipitated using 5 μ g/ml of either a polyclonal goat anti-AhR antibody (lanes 2,3) or a control goat IgG (lanes 4,5). Of 30 μ l of resulting antibody-protein A-sepharose eluates, 20 μ l were subjected to immunoblot analysis with RelA-specific antibody (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (30 μ g protein) was analysed as a positive control (lane 1) (TC: total cell lysate). The position of the 65 kDa RelA protein is indicated. (b) Cytosolic (200 μ g from 1.5 mg total) or nuclear (100 μ g from 330 μ g total) proteins from Hs578T cells were immunoprecipitated using 5 μ g/ml of either a polyclonal rabbit RelA-specific antibody (sc-372) (lanes 2,4) or a control rabbit IgG (lanes 3,5). Of 50 μ l of resulting antibody-protein A-sepharose eluates, 30 μ l were subjected to immunoblot analysis with polyclonal goat anti-AhR antibody (sc-8088) (Cyt: cytosolic immunoprecipitate; Nuc: nuclear immunoprecipitate). Total cell lysate (40 μ g protein) was analysed as a positive control (lane 1) (TC: total cell lysate). The position of the AhR protein is indicated. (c) Total cell proteins (100 μ g) from *T7-pcDNA3-AhR*- or *T7-pcDNA3*-transfected MCF-10F cells were immunoprecipitated using 5 μ g/ml of either normal rabbit IgG as a negative control (lane 1) or RelA-specific antibody (lanes 3,4). The resulting antibody-protein A-sepharose eluates were subjected to immunoblot analysis with T7 epitope-specific antibody, as described above. Total cell lysate (30 μ g protein) was analysed as a positive control (lane 2). The 97–100 kDa T7-AhR product is indicated.

transfected with the parental *T7-pcDNA3* DNA, as control. Total cell lysates were prepared, and either immunoprecipitated with a RelA-specific antibody or an aliquot run directly on the gel. The resulting

immunoblot was probed with a T7 epitope-specific antibody. A protein of the size expected for T7-tagged AhR, i.e. 97–100 kDa, was recognized in total cell extracts from cells transfected with the *T7-pcDNA3-AhR* vector (Figure 1c, lane 2). Similarly a 97 kDa AhR protein was detected following co-precipitation with RelA-specific antibody (lane 3), whereas no protein was detected following 'immunoprecipitation' with control rabbit IgG (lane 1). No anti-T7 antibody-reactive protein was detected in extracts from cells transfected with the parental vector (lane 4). Furthermore, AhR protein was not detected in T7-AhR-specific immunoblots of *T7-pcDNA3*-transfected MCF-10F cell extracts precipitated with a p50-specific antibody (data not shown). Similar results were obtained with transfected Hs578T cells (not shown). Overall, these results indicate that the RelA, but not p50, and the AhR are physically associated within Hs578T and MCF-10F cells.

RelA and AhR cooperate to activate the c-myc promoter in non-malignant MCF-10F cells

To determine whether AhR and NF- κ B/Rel can function cooperatively, we examined the effects of *AhR* and *RelA* co-transfection on the *c-myc* promoter, a transcriptional target of classical NF- κ B (La Rosa *et al.*, 1994). Since we have found that endogenous AhR levels decrease in cells as the cultures reach confluence (SAQ and DHS, unpublished observations), all transfections were performed with confluent cultures. In addition, in order to maximize conditions for observing RelA-AhR transcriptional cooperation, we first titrated the dose of expression vector transfected such that minimal augmentation of reporter activity would be observed. Addition of 1 μ g of the *pEVRF-p65* expression plasmid increased the transcriptional activity of the p1.6 Bgl promoter \sim 20-fold (data not shown), consistent with the potent role of RelA in transactivation of the *c-myc* promoter (La Rosa *et al.*, 1994). When the level of RelA expression plasmid was lowered eightfold to 0.125 μ g, a 2.4 ± 0.5 -fold increase in transactivation of the murine *c-myc* promoter was observed (Figure 2a). This amount (0.125 μ g) was selected for future transfections. Minimal transcriptional activity was observed when MCF-10F cells were transfected with p1.6 Bgl alone (Figure 2a). This is consistent with the observation that *c-myc* gene transcription is minimal in cells at confluence. Transfection of 2 or 4 μ g of AhR alone had no effect on the transcriptional activity of the p1.6 Bgl promoter. However, when 0.125 μ g *pEVRF-p65* were co-transfected with 2 μ g *pcDNA3-AhR* expression plasmid, a 6.2 ± 0.3 -fold induction of *c-myc* promoter activity was seen. Upon co-transfection of 0.125 μ g *pEVRF-p65* with 4 μ g *pcDNA3-AhR* expression plasmid the fold induction was 8.0 ± 0.2 . Using the latter conditions, an average fold induction in three separate experiments of 5.6 ± 2.1 -fold ($P < 0.01$) was obtained. Thus, RelA and AhR cooperate to significantly increase *c-myc* promoter activity in MCF-10F cells.

We next asked whether the increase in transactivation of the *c-myc* promoter was mediated by the NF- κ B elements located upstream of the promoter and/or within exon 1 (URE and IRE, respectively) (Duyao *et*

al., 1990; Kessler *et al.*, 1992b). A transfection experiment, similar to that described above, was performed using the p1.6 Bgl double mutant (p1.6 Bgl dbl mut) reporter plasmid, in which the URE and IRE NF- κ B sites have been mutated so that the promoter can no longer be transcriptionally activated by classic NF- κ B (Duyao *et al.*, 1992; Kessler *et al.*, 1992a; La Rosa *et al.*, 1994). In the absence of exogenous RelA or AhR, the p1.6 bgl double mutant displayed about one half of the activity of the wild type p1.6 Bgl reporter (Figure 2a). This modest decrease in activity of the mutant *vs* wild type p1.6 Bgl reporter construct is consistent with the low levels of RelA/p50 complexes present in the MCF-10F cells (Sovak *et al.*, 1997). Ectopically expressed RelA in *pEVRF-p65* transfected cells was unable to transactivate the mutant construct, consistent with our previous findings (La Rosa *et al.*, 1994). Furthermore, co-transfection with 4 μ g *pcDNA3-AhR* and 0.125 μ g *pEVRF-p65* did not significantly affect the activity of the mutated *c-myc* promoter (Figure 2a). Taken together these findings indicate that RelA and AhR function cooperatively to transactivate the *c-myc* promoter via binding at the URE and/or IRE NF- κ B elements.

RelA and AhR activate the c-myc promoter in Hs578T cells

We next asked whether RelA and AhR can activate the *c-myc* promoter in a human malignant breast cancer cell line by performing similar co-transfection analyses with Hs578T cells. In these cells the basal activity of the p1.6 Bgl promoter was notably higher than observed in transfected MCF-10F cells (Figure 2b). This result likely reflects the higher transfection efficiency of Hs578T cells (20–30% *vs* 5% for MCF-10F cells), and potentially the higher endogenous levels of nuclear NF- κ B/Rel proteins in these malignant cells (Sovak *et al.*, 1997). Interestingly, the activity of the p1.6 Bgl reporter plasmid increased in a dose-dependent fashion with transfection of increasing levels of AhR expression plasmid alone. Following transfection with 4 μ g of *pcDNA3-AhR*, CAT activity was 2.1 ± 0.4 -fold higher than basal levels. This result may be due to the effect of relatively high levels of constitutively active endogenous RelA protein present in these cells (see below). When a suboptimal dose (0.25 μ g) of *pEVRF-p65* plasmid alone was added, no apparent change in p1.6 Bgl activity was seen. However, co-transfection of both the RelA and AhR expression plasmids resulted in induction of a higher level of *c-myc* promoter activity than was seen following transfection of either plasmid alone (Figure 2b). Specifically, a 3.1 ± 0.3 -fold induction of the *c-myc* promoter activity was observed following co-transfection with 4 μ g *pcDNA3-AhR* and 0.25 μ g *pEVRF-p65* expression plasmids. The fact that the cooperative effects seen following AhR and RelA plasmid co-transfections in Hs578T cells were not as great as those seen in co-transfected MCF-10F cells may have been due to the higher level of background activity in the former cells, as well as the modest induction of reporter activity following transfection with 4 μ g AhR expression plasmid alone in Hs578T cells.

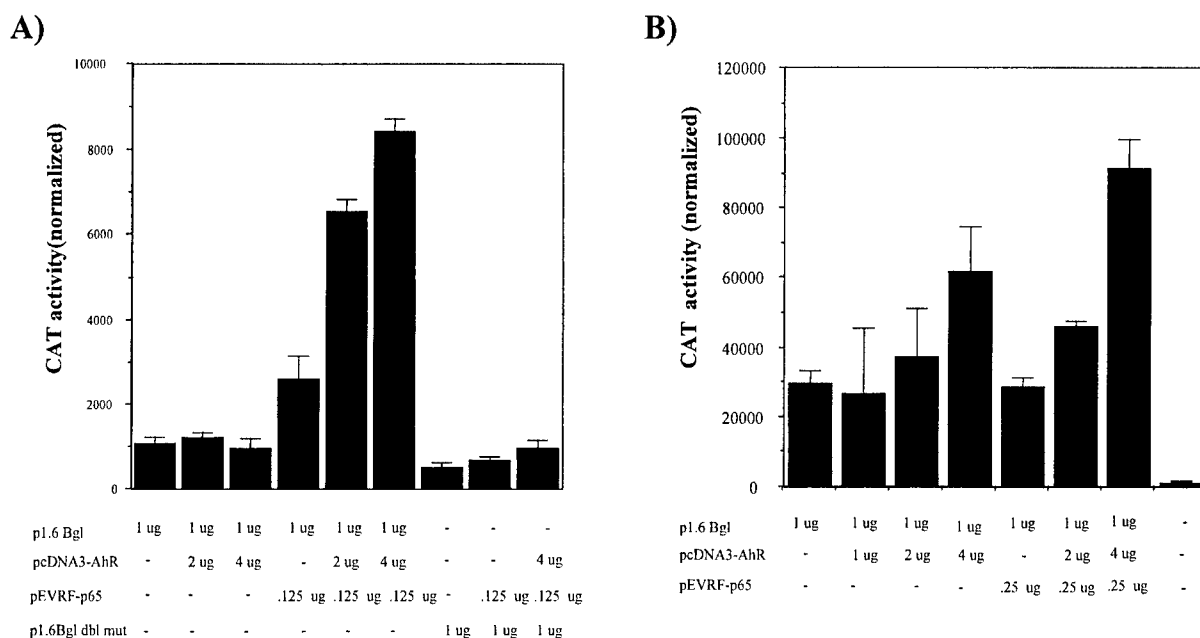


Figure 2 RelA and AhR cooperate to transactivate the wildtype p1.6 Bgl, but not the p1.6 Bgl dbi mut, *c-myc* promoter construct. (a) Confluent MCF-10F cells (~200 000 cells in 35 mm² dishes) were transiently transfected, in duplicate, with either 1 µg p1.6 Bgl or p1.6 Bgl dbi mut, and 0, 2, or 4 µg pcDNA3-AhR (murine AhR) expression vector in the absence or presence of 0.125 µg pEVRF-p65 (RelA expression) plasmid using 7 µl FUGENE reagent. In each transfection, 1 µg of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6 µg by addition pcDNA3 plasmid (parent vector for pcDNA3-AhR). Transfected cells were harvested after 24 h in reporter lysis buffer, and analysed for CAT and luciferase activity. CAT activities are presented normalized for transfection efficiency, using the luciferase activity. (b) Confluent Hs578T breast cancer cells were transiently transfected, in duplicate, with 1 µg of p1.6 Bgl plus 0, 1, 2, or 4 µg of pcDNA3-AhR, in the absence or presence of 0.25 µg of pEVRF-p65 plasmid using 5 µl of FUGENE reagent. In each transfection, 0.5 µg of TK-luciferase plasmid was added and total DNA was maintained at 6 µg by addition of the appropriate amounts of pcDNA3 plasmid. After 24 h, cells were harvested and analysed for CAT and luciferase activities and protein levels. Values were normalized to protein levels because the TK-luciferase activity was not appreciable in these cells at confluence

AhR/RelA complexes bind to the URE NF-κB element

To determine whether the AhR and RelA proteins are able to associate with the NF-κB binding elements in the *c-myc* gene, electrophoretic mobility shift assays (EMSA) were performed using an oligonucleotide containing the NF-κB upstream regulatory element (URE) as probe. To enhance AhR and RelA expression, nuclear extracts from transfected Hs578T cells were used. (Hs578T cells were selected rather than MCF-10F cells because of the better transfection efficiency obtained with this line.) Confluent cultures of Hs578T cells were transfected using FUGENE with pcDNA3-AhR vector DNA in the absence or presence of pEVRF-p65 RelA expression plasmid. As an additional control, cells were transfected with empty parental pcDNA3 vector alone. Nuclear extracts, prepared 24 h post-transfection, were then used in EMSA. Since an AhR/RelA complex might not bind DNA with the same affinity as typical NF-κB complexes, a lower dI:dC concentration was used to reduce the likelihood of competing away a specific binding complex. In control cells transfected with only parental pcDNA3, a major band migrating with the mobility of classic NF-κB was detected (labeled as band 1 in Figure 3a). No change in the binding pattern was seen upon transfection with pcDNA3-AhR. When nuclear extracts from cells co-transfected with pEVRF-p65 and pcDNA3-AhR expression plasmids were used, both the putative classic NF-κB band

and a novel upper band (labeled 'N') were seen (Figure 3a). The intensity of band 1 increased. Equal loading of the lanes was confirmed in EMSA for an Oct-1 probe (data not shown). Addition of fourfold or 20-fold molar excess wild type URE oligonucleotide successfully competed away complexes represented in both bands, whereas addition of similar amounts of mutant URE oligonucleotide, having the same two G to C conversions as in the p1.6 Bgl dbi mut construct (Duyao *et al.*, 1992), failed to compete (Figure 3b).

To determine the identity of the subunits found in the two specific binding complexes, supershift EMSA was performed using polyclonal rabbit antibodies raised against either RelA or the AhR. Addition of the AhR antibody specifically ablated band N without significantly changing the migration pattern of band 1 (Figure 4a). Antibody alone plus probe did not yield a similar complex (Figure 4c). Furthermore, the AhR antibody had no effect on binding of nuclear proteins to an Oct-1 sequence (data not shown). Addition of the RelA-specific antibody (sc-372X) clearly ablated formation of both band 1 and band N (Figure 4b,c). Addition of a second RelA-specific antibody #1226 (kindly provided by N Rice) similarly reduced formation of both band 1 and band N (data not shown). In contrast, addition of an antibody against the p50 subunit reduced band 1 and ablated a minor band below (Figure 4b). An equivalent amount of a rabbit polyclonal antibody against an irrelevant protein

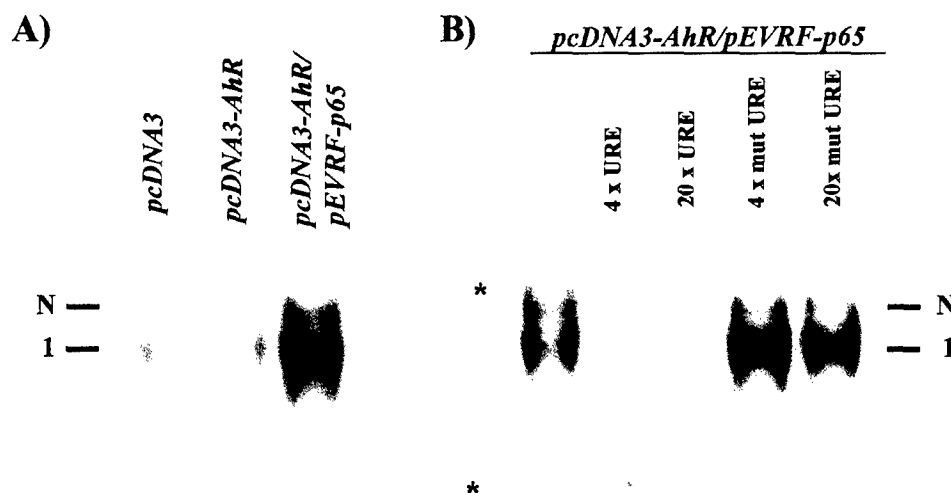


Figure 3 Expression of RelA and AhR yields a novel URE NF- κ B element binding complex. (a) Co-transfection with AhR and RelA expression vectors leads to formation of a novel complex. Confluent cultures (100 mm² dishes) of Hs578T cells were transfected with either 52 μ g *pcDNA3* empty vector, or 50 μ g *pcDNA3-AhR* in the absence or presence of 2 μ g *pEVRF-p65* expression plasmid using 70 μ l FUGENE reagent. After 24 h, nuclear proteins were isolated using the method of Dignam *et al.* (1983), and subjected to EMSA for NF- κ B binding. N indicates position of a new complex; 1, indicates position of a previously observed major complex. (b) Competition EMSA confirms the specificity of the major bands. Nuclear extracts of Hs578T cells co-transfected with *pcDNA3-AhR* and *pEVRF-p65* were pre-incubated with either 4- or 20-fold molar excess unlabeled wildtype (URE) or mutant (mt URE) URE prior to the 30 min incubation reaction with the radiolabeled URE. Two nonspecific bands were identified and marked with an asterisk (*)

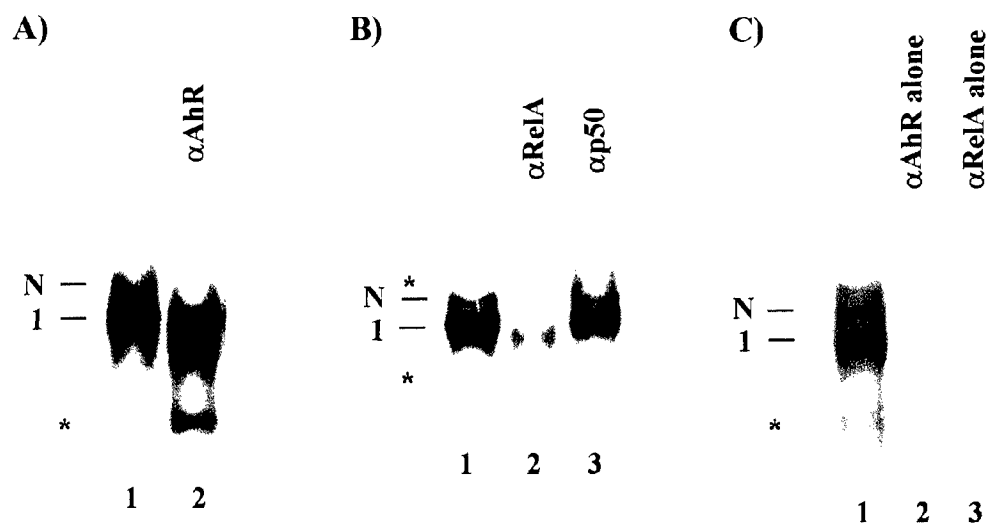


Figure 4 Novel NF- κ B binding complex contains AhR and RelA protein. Nuclear extracts from the AhR and RelA expression vector co-transfected cells, prepared as described above in Figure 3, were incubated with the URE probe. Following a 30 min binding reaction, antibodies were added as indicated, the reactions incubated for an additional 1 h, and subjected to EMSA. Alternatively as control, antibodies were added to the probe in the absence of extract, and the mixture incubated as above, and subjected to EMSA. (a) Extracts were incubated in the absence (lane 1) or presence of 1 μ l AhR-specific antibody (BioMol #SA-210) (lane 2) and subjected to EMSA. Specific binding complexes are indicated as band 1 and band N, as above; nonspecific bands are marked with an asterisk (*). (b) Extracts were incubated in the absence (lane 1) or presence of either 1 μ l RelA-specific antibody (sc-372X) (lane 2) or 1 μ l p50-specific antibody (sc-114) (lane 3), and processed as above. (c) Extract was incubated in the absence of antibody and EMSA performed, as above (lane 1). Alternatively either 1 μ l AhR-specific antibody (BioMol #SA-210) (lane 2) or 1 μ l RelA-specific antibody (sc-372X) (lane 3) was incubated with the probe alone, and subjected to EMSA

(YY1, sc-281-X) failed to alter binding to the URE (data not shown). Thus, band 1 contains RelA and p50 proteins, and most likely represents binding of classical NF- κ B heterodimers (RelA/p50). Based on its migration, the minor lower band likely consists of p50 homodimers. Finally, band N contains both RelA and AhR proteins.

AhR and RelA induce the endogenous c-myc gene

To verify that the affects of AhR and RelA can be seen on chromosomal *c-myc* genes, co-transfection analysis was performed. Cultures of MCF-10F cells at 70% confluence were transfected with *pEVRF-p65* or *T7-pcDNA3-AhR* vector DNA alone or in combination.

Whole cell extracts were prepared and subjected to immunoblot analysis for c-Myc and β -actin protein, which confirmed equal loading (Figure 5). Using densitometry of this and a duplicate experiment, an increase in c-Myc level of 3.1 ± 0.00 -fold and 2.75 ± 0.05 -fold, respectively upon expression of RelA or AhR alone compared to control vector DNA was measured. An increase in c-Myc expression of 9.5 ± 3.2 -fold was observed upon co-transfection of both *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs into MCF-10F cells. Thus, while increases in the level of c-Myc protein were seen upon transfection of MCF-10F cells with either vector alone, a greater induction was seen upon transfection of the combination of *pEVRF-p65* and *T7-pcDNA3-AhR* vector DNAs. These results confirm the ability of RelA and AhR to cooperate in activation of the *c-myc* gene.

Discussion

Here we show the physical and functional association of the RelA subunit of NF- κ B and AhR in transactivation of the *c-myc* gene in breast epithelial cells. Specifically, RelA and AhR were physically associated in malignant Hs578T breast cancer. Using transfection analysis, RelA and AhR cooperated to transactivate the *c-myc* promoter in non-malignant MCF-10F mammary epithelial cells and to a lesser extent Hs578T cells. Furthermore, RelA and AhR enhanced endogenous c-Myc protein levels in MCF-10F cells. As judged by transfection and mobility shift analyses, the RelA and AhR proteins formed a novel complex that bound to the wild type but not mutant NF- κ B element of the *c-myc* gene. We postulate it is this complex, binding via the NF- κ B element, that transactivated the *c-myc* promoter. Co-transfection of vectors that express AhR and RelA proteins with a wild type *c-myc* promoter-reporter construct, but not with a promoter construct mutated in the NF- κ B binding URE and IRE sites, led to increased levels of *c-myc* promoter transactivation. In contrast, cooperation between AhR and RelB or c-Rel subunits of NF- κ B/Rel in transactivation of the *c-myc* gene was not

observed in similar transfection analysis (data not shown). Furthermore, the novel transcription factor complex did not appear to contain the p50 subunit. Consistent with these findings, RelA but not p50 was found to specifically interact with the AhR in murine hepatoma cells (Tian *et al.*, 1999). Thus, based on the relative mobility in EMSA, and the identified presence of both RelA and AhR in the novel complex, our results suggest that the RelA and AhR bind the URE as a heterotypic dimer composed of one subunit of each protein.

Recently, we demonstrated that rodent and human mammary tumors are typified by aberrant activation of NF- κ B/Rel (Sovak *et al.*, 1997) and overexpression of AhR (Trombino *et al.*, 2000). These tumors are often also characterized by overexpression of *c-myc* (Berns *et al.*, 1992; Borg *et al.*, 1992; Pavelic *et al.*, 1991; DWK and GES, unpublished observations). While in some tumors *c-myc* genes were present in large copy numbers, in other cases overexpression of c-Myc protein was seen without gene amplification (Pavelic *et al.*, 1991). The ability of AhR to cooperate with RelA to transactivate promoters through NF- κ B elements suggests a novel mechanism for regulation of *c-myc* gene expression.

RelA protein interactions with other transcription factors have been found to lead either to induction (Bassuk *et al.*, 1997; Shen and Stavnezer, 1998) or repression (Wissink *et al.*, 1997; Ferrier *et al.*, 1999) of gene transactivation. In most cases, transcription complex-DNA association involves binding sites for both NF- κ B and the partner transcription factor, which are in close proximity to one another (Shen and Stavnezer, 1998; Dickinson *et al.*, 1999). However, neither a consensus XRE, which would bind AhR/ARNT complexes, nor an AhR binding half site (5'-CGTC-3') (Dickinson *et al.*, 1999) are present in close proximity to either the URE or the IRE NF- κ B elements in the murine *c-myc* gene. In addition, a 10- or 50-fold molar excess of cold XRE did not compete successfully for AhR/RelA-URE binding (data not shown), suggesting that DNA domains typically bound by an AhR/ARNT complex are not required for AhR/RelA-URE binding. Consistent with this observation, competition EMSA with oligonucleotides mutated at additional bases within the core NF- κ B element failed to successfully compete for binding (data not shown).

Our findings differ significantly from those reported by Tian and coworkers (1999) who observed repression of RelA transactivation by AhR in a murine cell line. Several explanations may be given for these differences. Tian *et al.* (1999) used a multimerized consensus NF- κ B element (5'-GGCAGGGGAATTCCCC-3') construct in their studies, while we employed a *c-myc* promoter construct. Of note, the core sequence of the consensus binding element differs significantly from that found in the two NF- κ B elements within the *c-myc* gene (Duyao *et al.*, 1990; Kessler *et al.*, 1992b). Interestingly, no new binding complex was seen with the NF- κ B consensus element (Tian *et al.*, 1999), whereas EMSA with the *c-myc* URE NF- κ B element revealed a novel AhR/RelA-containing band, consistent with the functional cooperation in cells co-transfected with AhR and RelA expression vectors. If DNA binding is sequence specific, then only a subset of NF- κ B element-containing genes may be affected by

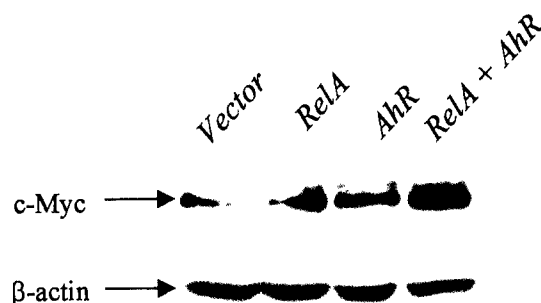


Figure 5 RelA and AhR cooperate to induce the endogenous *c-myc* gene in MCF-10F cells. Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 μ g *pEVRF-p65* or 20 μ g *T7-pcDNA3-AhR* DNA alone or in combination with 30 μ l FUGENE transfection reagent. Total transfected DNA was maintained at 24 μ g by addition of *pcDNA3* plasmid. Alternatively, cells were transfected with *pcDNA3* plasmid DNA alone (Vector). After 48 h, cells were harvested and samples of whole cell extracts (40 μ g) subjected to immunoblot analysis for c-Myc (786-4) and β -actin (AC-15) proteins

AhR/RelA binding. Furthermore, the *c-myc* promoter likely contains elements capable of binding other potential cooperating transcription factors. Important differences also reside within the cell types used, i.e. murine hepatoma and COS-7 cells *vs* human mammary cell lines. Interestingly, NF- κ B failed to transactivate the *c-myc* promoter in normal mouse hepatocytes (Bellas and Sonenshein, 1999), while it effectively induced the promoter in breast cancer cells (Sovak *et al.*, 1997), suggesting that binding to overlapping elements within the *c-myc* gene ablated the ability of NF- κ B to bind in hepatocytes.

Different subunits of the NF- κ B/Rel family have been shown to interact with members of other protein families (Bassuk *et al.*, 1997; Shen and Stavnezer, 1998; Wissink *et al.*, 1997; Ferrier *et al.*, 1999; Dickinson *et al.*, 1999; Stein *et al.*, 1993; Raj *et al.*, 1996; Kalkoven *et al.*, 1996; Na *et al.*, 1999). In many of these cases, the associations are fairly specific for the RelA subunit, e.g., with glucocorticoid and progesterone receptors (Wissink *et al.*, 1997; Kalkoven *et al.*, 1996) and the YB-1 protein (Raj *et al.*, 1996). In contrast, Stat6 (Shen and Stavnezer, 1998), C/EBP (Stein *et al.*, 1993), and retinoid X receptor (Na *et al.*, 1999) functionally interact with both RelA and p50. Therefore, it is not unusual that only cooperation between AhR and RelA was detected in HMECs. Interestingly, a similar functional interaction of RelA with the progesterone receptor was noted in that p50 and c-Rel subunits failed to affect the transcriptional activity of the activated PR on a progesterone responsive element construct (Kalkoven *et al.*, 1996). The Rel homology region (RHR) is known to be important both for dimerization of NF- κ B/Rel subunits (Grimm and Bacuerle, 1993), as well as for interaction with many of these other proteins (Wissink *et al.*, 1997; Stein *et al.*, 1993). While some AhR domains involved in AhR binding to proteins, such as ARNT, hsp90, and the immunophilin-like ARA-9 protein have been evaluated (Perdew and Bradfield, 1996; Meyer *et al.*, 1998; Okey *et al.*, 1994; Carver *et al.*, 1998), those required for AhR dimerization with other proteins, e.g. Rb (Ge and Elferink, 1998), have not been defined. The exact domains mediating the interactions between AhR/RelA and binding of the putative heterodimeric complex to the *c-myc* promoter are under investigation.

The human *c-myc* gene has been found to contain consensus XRE elements. Since our efforts were focused on the potential effects of AhR/RelA interactions, a *c-myc* promoter construct that does not contain these elements was used to reduce complications with effects of AhR alone. Finally, it should be noted that *c-myc* promoter activation following transfection with AhR- and RelA-encoding plasmids and AhR-RelA dimerization in the nuclei of non-transfected cells occurred in the absence of exogenous AhR ligands. These results suggest constitutive AhR activity in mammary tumor cell lines. This hypothesis is strongly supported by constitutive nuclear AhR expression (Chang and Puga, 1998; Singh *et al.*, 1996), and constitutive AhR-mediated transcriptional activity (Chang and Puga, 1998; Ma and Whitlock, 1996) in mouse hepatoma, monkey kidney, and human epithelial carcinoma cell lines. Furthermore, we have recently demonstrated constitutive nuclear AhR expression and high levels of an AhR-regulated gene, *CYP1B1*, in rat

mammary tumors (Trombino *et al.*, 2000). While these results support a role for constitutive AhR activation in tumorigenesis, organ defects observed in AhR^{-/-} mice suggest a role for developmentally regulated AhR activation in organogenesis (Hushka *et al.*, 1998; Fernandez-Salguero *et al.*, 1995; Lahvis and Bradfield, 1998; Abbott *et al.*, 1999; Robles *et al.*, 2000). The endogenous signals that induce AhR activity, and the extent to which these AhR activities are modulated by exogenous AhR ligands remain to be elucidated.

Materials and methods

Cell growth and treatment conditions

MCF-10F is a human mammary epithelial cell line established from a patient with fibrocystic disease, which does not display malignant characteristics (Calaf and Russo, 1993). The Hs578T tumor cell line was derived from a mammary carcinosarcoma and is epithelial in origin (Hackett *et al.*, 1977).

Synthesis of AhR expression construct

Full length AhR cDNA was PCR amplified using the pMu-AhR plasmid (kindly provided by Dr C Bradfield, University of Wisconsin, Madison, WI, USA) as template, with the following primers carrying *Xba*I restriction sites: sense 5'-CTA GTC TAG ACC ATG AGC AGC GGC GCC AAC-3'; anti-sense 5'-CTA GTC TAG AAA GCT TAG TAT CGA ATT-3'. The AhR cDNA was amplified with Pfu Turbo polymerase (Stratagene, La Jolla, CA, USA). The PCR product was gel purified and subcloned into the *Xba*I site of the *T7-pcDNA3* plasmid constructed by linking the DNA coding for the 11 amino acid leader peptide of the T7 major capsid protein (digested out from the pTOPE pET translation vector (Novagen, Madison, WI, USA) to the *Bam*HI site of *pcDNA3* (Invitrogen, Carlsbad, CA, USA). Proper AhR and *T7-pcDNA3* directional cloning was confirmed by restriction analysis and DNA sequencing.

Transfection and immunoprecipitation analysis

Cells were transfected in 100 mm² culture plates with 5 μ g of *T7-pcDNA3-AhR* or *T7-pcDNA3* mixed with 6 μ l FUGENE transfection reagent (Boehringer Mannheim, Indianapolis, IN, USA), according to the manufacturer's instructions. After 36 h, cells were rinsed with cold PBS. For total cell lysates, transfected or untransfected cells were lysed in 1 ml immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 2 μ g/ml leupeptin; 2 μ g/ml aprotinin; 5 μ g/ml phenylmethylsulfonyl fluoride) containing 1% IGEPAL CA-630 detergent (Sigma Chemical Co., St. Louis, MO, USA) for 20 min on ice, and centrifuged at 14 000 r.p.m. for 10 min. Alternatively, cytosolic and nuclear fractions were prepared essentially as described (Pollenz *et al.*, 1994). Aliquots were incubated for 1 h with 5 μ g/ml polyclonal rabbit anti-RelA/p65 antibody, normal rabbit IgG, polyclonal goat anti-AhR antibody or normal goat IgG (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and immunoprecipitates collected and washed using protein A-sepharose beads. Eluted proteins were subjected to electrophoresis and immunoblot analysis, as described previously (Yamaguchi *et al.*, 1997a,b). Blots were probed with HRP-anti-T7-epitope tag antibody (Novagen), anti-RelA antibody (sc-372, Santa Cruz Biotechnology), or polyclonal goat anti-AhR antibody (sc-8088, Santa Cruz Biotechnology) for 1 h at room temperature. After thorough washing, the membranes were treated for another 45 min with goat anti-rabbit-HRP antibody for RelA-specific

immunoblotting or with anti-goat IgG-HRP antibody for AhR-specific immunoblotting. Membranes were developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA, USA) after washing three times with TBS containing 0.05% Tween (Sigma).

Transfection and immunoblot analysis

Cultures of MCF-10F cells, at 70% confluence, were transiently transfected with 4 µg *pEVRF-p65* or 20 µg *T7-pcDNA3-AhR* DNA alone or in combination with 30 µl FUGENE transfection reagent. Total DNA transfected was maintained at 24 µg by addition of *pcDNA3* plasmid. After 48 h, cells were rinsed with cold PBS, and harvested in lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 150 mM NaCl; 0.5 mM DTT; 2 µg/ml aprotinin; 2 µg/ml leupeptin; 0.5 mM PMSF; 0.5% NP40). Whole cell extracts (WCE) were obtained by sonication, followed by centrifugation at 14 000 r.p.m. for 30 min. Samples (40 µg) of WCEs were subjected to electrophoresis and immunoblot analysis, as above. Blots were probed with rabbit anti-c-Myc antibody (786-4, a gift from S Hann, Vanderbilt University, Memphis, TN, USA), and mouse anti-β-actin monoclonal antibody (AC-15, Sigma).

Promoter activity analysis

Confluent cultures of MCF-10F or Hs578T cells were transiently transfected using FUGENE transfection, as above, with wild type p1.6 Bgl *c-myc* promoter CAT or a mutant *c-myc* promoter CAT reporter, termed p1.6 Bgl dbl mut vector, described previously (Duyao et al., 1992). These constructs contain -1141 to +513 bp of the murine *c-myc* promoter/exon1/upstream sequences, including the two NF-κB elements in either wild type or mutant versions, driving a chloramphenicol acetyl transferase (CAT) reporter gene (Duyao et al., 1992), and does not contain XREs. Vectors *pcDNA3-AhR*, encoding murine AhR (Dolwick et al., 1993) and *pEVRF-p65*, encoding murine RelA protein (kindly provided by R Sen, Brandeis University, Waltham, MA, USA) were co-transfected, as indicated. In each transfection, 1 µg of TK-luciferase plasmid was added as an internal control for normalization of transfection efficiency. Total DNA transfected was maintained at 6 µg by addition of *pcDNA3* plasmid (parent vector for *pcDNA3-AhR*). Transfected cells were harvested after 24 h in reporter lysis buffer,

and analysed for CAT and luciferase activity, as described previously (Sovak et al., 1997; Dolwick et al., 1993).

EMSA

Nuclear extracts were prepared from breast cancer cells by a modification of the method of Dignam et al. (1983), and oligonucleotides probes radiolabeled essentially as we have described previously (Sovak et al., 1997). The sequence of the URE NF-κB-containing oligonucleotide from the *c-myc* gene (Duyao et al., 1990) is as follows: 5'-GATCCAAGTCCGG-GTTTTCCCAACC-3', where the underlined region indicates the core binding element. The mutant URE has a two G to C base pair conversion, indicated in bold, blocking the NF-κB/Rel binding (Duyao et al., 1990): 5'-GATCCAAGTCCG**GCCTTTT**CCCAACC-3'. A slight modification of the usual NF-κB binding reaction (Sovak et al., 1997) was used. ³²P-labeled oligonucleotide (20 000–25 000 c.p.m.) was incubated with 2.5 µg of nuclear extract, 5 µl sample buffer (10 mM HEPES, 4 mM DTT, 0.5% Triton X-100, and 2.5% glycerol), 0.1 µg poly dI-dC as nonspecific competitor, and the salt concentration adjusted to 100 mM using buffer C. The reaction was carried out at room temperature for 30 min, and DNA/protein complexes were separated, as previously described (Sovak et al., 1997). Where indicated, antibodies were added after the binding reaction and the mixture incubated for 1 additional hour. Antibodies used include: anti-RelA subunit, sc-372X from Santa Cruz Biotechnology and #1226, kindly provided by N Rice (NCI, Frederick, MD, USA); anti-p50 subunit, sc-114 from Santa Cruz Biotechnology; anti-AhR, #SA-210 from BioMol (Plymouth Meeting, PA, USA).

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